Endotoxins Standards and Their Role in Recovery Studies: The Path Forward

Karen Zink McCullough
MMI Associates

Radhakrishna Tirumalai, Ph.D.
United States Pharmacopeial Convention

David Hussong, Ph.D.
ValSource, LLC

James Akers, Ph.D.
Akers Kennedy Associates

Dennis Guilfoyle, Ph.D.
Johnson and Johnson

Robert Mello, Ph.D.
Mello PharmAssociates, LLC

Donald Singer
Glaxo Smith Kline

The USP General Chapters – Microbiology Expert Committee (EC) is the steward in the United States of harmonized Chapter <85>, "Bacterial Endotoxins Test", or BET (USP 2016a). The EC has closely followed the Low Endotoxin Recovery ("LER") discussion and publications since the interference was first described in 2013 (Chen and Vinther, 2013). It is important to note that the use of RSE and the BET for hold time studies is a non-compendial activity. However the concerns raised by some are important given the potential implications relative to the validity of the compendial BET assays for the release of "LER" products, and most importantly, patient safety.

Summary

The initial studies of the "LER" phenomenon describe a sudden and irreversible loss of "endotoxin activity" when Control Standard Endotoxin (CSE), in the form of chemically extracted and purified lipopolysaccharide (LPS) was added to an undiluted biological product containing a protein formulated in a chelating buffer with polysorbate. This observation led some readers, including some regulators, to assume that if the recovery of activity of this CSE is suppressed, then the activity of contaminating and native endotoxins must also be similarly affected. This assumption, made in the absence of supporting data, led some to an unsubstantiated conclusion that this loss of CSE activity equated to potential false negative LAL drug product release test results using <85>, a possible release of contaminated product into the marketplace, and therefore a possible patient safety concern. As a result, the United States Food and Drug Administration (FDA) has informed companies intending to submit new Biological License Applications (BLA) for an "LER" product" that they must either demonstrate that they can overcome or correct for this interference seen with the CSE LPS in undiluted product or default to USP <151>, "Pyrogen Test", also known as the Rabbit Pyrogen Test, to release product.

The USP Expert Committee has proposed the concept of an alternate CSE preparation that we believe is a scientifically valid option as one possible resolution to this "LER" interference problem.

Some Background

In 2012, FDA retired a long standing Guidance document entitled, "Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (FDA, 1987), and replaced it with a new guidance document entitled, "Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers" (FDA, 2012). The genesis of the concept of a "hold time" as it relates to endotoxins recovery studies might be related to FDA's published concerns about holding product under inappropriate conditions and/or improper sample mixing prior to testing and release (Guilfoyle, et al, 1989). These concerns were ultimately published as "Question 3" of the 2012 Questions and Answers document:

Question 3: Is sample storage and handling important?
Yes. The ability to detect endotoxins can be affected

by storage and handling. Firms should establish procedures for storing and handling (which includes product mixing) samples for bacterial endotoxins analysis using laboratory data that demonstrate the stability of assayable endotoxins content. Protocols should consider the source of endotoxins used in the study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins.

This seemingly simple question and its relatively short answer have precipitated a whole new set of questions related to "storage and handling" (i.e. "hold time") among Microbiologists in the parenteral industry:

1. What is "hold time"?
   a. Is "hold time" defined as FDA's original concern about endotoxins recovery between the completion of fill/finish activities (primary packaging) and testing?
   b. Is "hold time" a specified period of time that is built into the manufacturing process and described in the batch record to provide a required incubation or level of flexibility between processing steps?
   c. Is "hold time" essentially the entire process from the time that formulation takes place until the time that the product is tested or expires?
   d. Does "hold time" refer to conditions where the bulk is not sterile and where any Gram negative contamination could grow and produce endotoxins or does it also refer to hold times of sterile products where such proliferation and generation of endotoxins is not an issue?

2. What is "stability"?
   a. Is there a concern about the chemical or biochemical stability of contaminating endotoxin in a filled product?
   b. Is there a concern that contaminating endotoxin in a bulk product will somehow change, lose activity, and "self depyrogenate?"
   c. Is there a concern about LPS adsorbing to surfaces such as stoppers and vial walls that manifests as a loss of activity?

3. What are "assayable" endotoxins?

4. Is industry required to prospectively perform spiking studies to demonstrate the stability of activity during a "hold time" recovery study?

5. If so, what spiking analyte is appropriate?
   a. Is the calibration standard (Reference Standard Endotoxin, or RSE) an appropriate surrogate for contaminating endotoxins?

6. What does the Guidance's disclaimer, "*Protocols should consider the source of endotoxins used in the study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins*" mean?

Discussions with a number of people who participated in the drafting of the 2012 Guidance (including three authors of this article) have provided some insight into the original intent of Question 3.

- The initial definition of hold time was meant to be the time between the completion of fill/finish and compendial release testing.

- "Assayable endotoxins" was meant to describe endotoxin activity in products that are naturally contaminated.

- Stability means that the activity of endotoxins in a naturally contaminated product can be recovered (within the error of the assay) throughout its defined storage period and conditions ("hold time").

- And, yes, the authors did acknowledge that there could very well be a difference in the behavior of purified bacterial endotoxins (LPS reference standards) and native endotoxins in these hold time studies, suggesting that either a purified or a native analyte may be an appropriate spiking material.

Laboratories and some regulators have interpreted the answer to Question 3 to mean that prospective spiking studies are expected to measure recovery of activity across study-specific parameters including time, temperature and sample container. Since there have been no public or published reports of any safety issues associated with "LER", the focus of the discussion has now shifted from "What is "LER" and is it relevant to patient safety?" to "Assuming such relevance, what is an appropriate analyte to use for these recovery studies? Should laboratories use purified LPS prepared in a manner similar to the RSE or is it scientifically justified to use native sources of endotoxins?"

Endotoxins

Endotoxins are structural components of the Gram negative bacterial outer cell membrane. What makes the Gram negative outer membrane unique is the presence of lipopolysaccharide (LPS), an amphipathic molecule with a hydrophobic Lipid A end that is buried in the outer membrane of the bacterium, and a hydrophilic polysaccharide portion that extends beyond the outer membrane into the extracellular environment. The Lipid A part of the LPS molecule is the biologically active portion, which is recognized by immune cell receptors and can trigger a number of responses in mammals including fever (Pearson, 1985). Lipid A also initiates the Limulus Amebocyte Lysate (LAL) cascade.

Although endotoxins activity is extremely rare in marketed pharmaceutical products, if a laboratory does find evidence of endotoxins, it is always the native form of the LPS molecule that is the contaminant; purified LPS cannot and does not contaminate pharmaceutical products because it does not exist in nature. Purified LPS is chemically extracted from Gram negative outer membranes in the laboratory. On the other hand native contaminants are vesicles (blebs, outer membrane vesicles/OMV) that are naturally released from actively proliferating cells as part of the bacterial growth cycle, (Brogden and Phillips, 1988, Bonnington and Kuehn, 2016) as well as outer membrane fragments that are the result of disrupted cells. As an integral part of these vesicles and fragments, the LPS in native endotoxins is embedded in or associated with Gram negative outer membrane components including various proteins, phospholipids and lipoproteins (Brogden and Phillips, 1988, Bonnington and Kuehn, 2016).

USP Endotoxin RS (RSE)

USP Endotoxin RS (RSE) is the primary reference material used in the compendial BET tests <85>. The RSE is derived from *E. coli* strain 0113:H110K(-) (Rudbach, et al, 1976) that has been grown in a broth to produce a large quantity of native endotoxins. These native endotoxins are subsequently treated using the Westphal (hot phenol) extraction process to strip away the surrounding natural cell wall and outer membrane components. This preparation is further purified to produce bulk material for the RSE. This material, now commonly accepted and distributed by the World Health Organization, the United States Pharmacopeia, the European Pharmacopeia and the Japanese Pharmacopeia, is formulated in polyethylene glycol and lactose, components NOT associated with or found in native endotoxins. The current RSE is lyophilized, and each vial has an assigned activity

value of 10,000 endotoxin units (EU). (Poole, et al, 1997; Poole 2012).

FDA's very first RSE was a *Klebsiella pneumonia* LPS preparation and Europe's first Biological Reference Preparation (BRP) was a liquid preparation of *Salmonella abortus equii* LPS. Why then was *E. coli*, an organism that is an unlikely contaminant in parenteral products, chosen as the source of the primary standard for the LAL test?

It is important to understand that the current RSE was not chosen because it is "worst case" in terms of its relevance to the pharmaceutical industry, or the most difficult analyte to detect in a recovery study. Truth is, a number of early researchers looked at the relative potencies of LPS preparations from a range of Gram negative bacteria, and they generally found that *E. coli* LPS was "typical" in that it consistently had a mid-range potency (Rudbach, et al,1976; Firca and Rudbach, 1982; Weary and Pearson, 1982). The only thing special about this preparation is its history of use as a BET calibrant. As a "typical" LPS, it is no more or less representative to the pharmaceutical industry than endotoxins derived from other Gram negative species. It is also important to note that there is not a limitless supply of the current lot of RSE and a new standard supply will be required in the foreseeable future (Poole, et al, 2012).

Secondary Standard: Control Standard Endotoxin

The Control Standard Endotoxin (CSE) is, by definition, a secondary standard (USP, 1985). A definition of the CSE was provided in USP XXI:

> *"A Control Standard Endotoxin (CSE) is an endotoxin preparation other than the RSE that has been standardized against the RSE. If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters both for endotoxin quality and performance (such as reaction in the rabbit) and for suitability of the material to serve as a reference (such as uniformity and stability)."*

By this definition, any preparation of bacterial endotoxins other than the RSE, regardless of source, can be called a CSE as long as it is characterized and standardized against the RSE.

Although there is no requirement in this USP definition that a CSE must be either purified, formulated, lyophilized or of *E. coli* origin, all of the CSE preparations currently packaged in LAL test kits are, in fact, LPS extracted from various strains of *E. coli*. These preparations are further purified, most are further formulated, and all are lyophilized. Test kit CSEs are filled by weight (ng/vial), not by activity (EU/mL or EU/vial). Standardization of these preparations requires the calculation of their specific activities relative to the RSE, meaning that the EU/ng must be experimentally determined. Lysate manufacturers provide this standardization service, and report potency in EU/ng that is referenced to a matched set of lysate lot and CSE lot, allowing, of course, for inherent biological assay variability.

By contrast, a liquid CSE such as the original BRP has no potency because it has no determined weight. It is measured directly against the primary standard as a concentration of activity (EU/mL).

Comparison:

Table 1 compares the structure and properties of purified LPS and native endotoxins. For an excellent visual comparison in the form of electron micrographs of the two materials, see Brogden and Phillips (1988).

"LER", Control Standard Endotoxins, and Future Improvements

The initial choice of a CSE analyte in the form of purified LPS for recovery studies from final dosage forms and product stream depyrogenation studies is understandable, as this material is readily available from lysate manufacturers, comes standardized and matched to a specific lot of lysate reagent, and is used for the Positive Product Control (PPC) in the compendial BET. However, studies by Bolden, Platco, Dubczak and their colleagues as well as other scientists using both purified LPS and native endotoxins as spiking analytes in their "LER" formulations have demonstrated that while activity of purified LPS is suppressed, the activity of native endotoxins may not be similarly suppressed. More importantly, rabbit pyrogen studies on "LER" formulations spiked with purified LPS or native endotoxins demonstrated that the LAL test correctly predicted rabbit febrile responses for all native endotoxins. In other words, when the LAL response to purified LPS was suppressed because of "LER", so was the rabbit febrile response (Bolden, et al, 2015).

What causes this suppression of activity in these "LER" product formulations? Because of their amphipathic nature, purified LPS molecules in aqueous solution tend to aggregate, forming micelles, ribbons, and other three-dimensional conformations. Data suggest that there is an optimal aggregation state for

Purified LPS	Native Endotoxins
Biologically active portion is Lipid A that is stripped of its natural associated cell membrane components	Biologically active portion is Lipid A existing in a natural state that is surrounded by components that are part of native Gram negative outer membrane.
Extracted, usually by a hot phenol extraction (Westphal) and further purified. It is ultimately formulated with chemicals that are not native to the cell membrane, usually polyethylene glycol and a sugar, prior to lyophilization	Exist as naturally generated and free floating outer membrane fragments and vesicles (blebs). LPS is embedded in or associated with cell membrane components (proteins, porin, phospholipids and lipoprotein). There is no extraction, no purification, and no further chemical formulation.
LPS molecules form aggregates in aqueous solution, with the extent of the aggregation dependent on the formulation of the matrix	Natives do not form aggregates in the same way as LPS since native LPS is embedded within cell membranes or vesicles
Readily adsorbs to surfaces	Does not readily adsorb to surfaces
Does not exist in nature and therefore cannot and does not contaminate parenteral products	Native endotoxins can be natural contaminants in water systems and raw materials, particularly those of natural origin. If not controlled, these natural contaminants can find their way into parenteral products.

Table 1

a purified LPS to be biologically active. The citrate or phosphate buffer/polysorbate formulation that is common to "LER" products, chelates divalent cations and destabilizes these aggregates, which results in smaller aggregates or unreactive LPS monomers (Ribi, et al, 1966; Hannecart-Prokorni, et al, 1973; Gutsman, et al 2007; Tsuchiya, 2014). The biochemistry of the native endotoxins, their position in the cell membrane, and their ability to adapt to environments with low divalent cation concentrations (chelating formulations) make them much less susceptible to the inactivating effects of chelation (Bonnington and Keuhn, 2016). Chelating agents can also affect the lysate's ability to initiate and sustain the LAL cascade, so in this sense, chelating agents can adversely affect the recovery of both purified and native LPS.

Referencing the data reported to date on the comparative recoveries of activity between native endotoxins and purified LPS in "LER" formulations and the correlation between loss of activity as measured by both the LAL test and the rabbit pyrogen test, the EC feels that it is scientifically valid to offer the laboratory a choice of a CSE in the form of native endotoxins. We recognize and understand the utility this new CSE or as an analyte in endotoxin activity recovery studies where the use of a native CSE more closely mimics a real life contamination event (USP 2016b; USP 2016c; Tirumalai, et al, 2016). It is important to note that this new offering will not replace either the current RSE as a primary calibration standard or the current CSEs as secondary standards. Rather, the proposed native CSE will provide an additional option for Microbiologists to use in their laboratory studies that is consistent with Question 13 from FDA's 2012 Guidance.

Question 13: **Are control standard endotoxins still acceptable for use in running bacterial endotoxins tests?**

Control standard endotoxins (CSEs) are endotoxin preparations other than the international or national reference standards that are traceable in their calibration to the international reference endotoxins standard. CSEs may be secondary or tertiary standards and are usually manufactured and certified by an LAL reagent manufacturer for use with a specific lot of reagent under defined assay conditions. CSEs have become an accepted source for preparation of standard curve calibrators and as assay controls, and have provided a cost saving to LAL users and helped to preserve the inventory of primary standards. FDA encourages the continued use of CSEs that are suitably calibrated to the international reference endotoxins standard.

On 27 May 2016, The United States Pharmacopeia posted an "Early Input" notification seeking comments on a proposed Naturally Occurring Endotoxin (NOE) Reference Standard, which is, by definition, a CSE (USP 2016c). Early input is intended to gauge interest in the offering and understand any potential obstacles early on. This request for feedback outlined the proposed characteristics for the new CSE:

- To assure wide ranging availability and consistency, the CSE will be universally available in large quantities and distributed by USP.
- To assure consistency in manufacturing and testing, each lot of the native CSE will be accompanied by a Certificate of Analysis stating its activity in EU/mL as calibrated against the RSE.
- To reduce lot-to-lot variability, the material will be:
  - Prepared from a well characterized bacterial species that has been isolated from parenteral environments.
  - A member of the larger family of Enterobacteriaceae is consistent with this definition and is closely related to the current RSE standard, which is prepared from *E. coli*.
  - Manufactured under GMP conditions, meaning that it will have a batch record, will be subject to change control, and will have appropriate specifications that will be recorded on the Certificate of Analysis.

The response to this request was overwhelmingly positive with 80% of respondents and experts in the field providing enthusiastic support for the Committee's proposal (Cooper, 2016).

The Importance of Process Control

Bacterial endotoxins don't spontaneously generate. Detecting endotoxins in any raw material, drug substance or drug product indicates that there were substantial numbers of proliferating Gram negative bacteria somewhere in the materials' history. While the introduction of an alternate CSE in the form of native endotoxins may help companies to successfully execute recovery and product stream depyrogenation studies, by far the best strategy for assuring patient safety is one that identifies sources of potential contamination by endotoxins and works to control their introduction.

The good news is that because of increased focus on process control including control of water systems and raw materials that can be prime sources of Gram negative bacterial contamination, coupled with the appropriate use of the BET as a monitoring tool for the effectiveness of control measures, our industry experiences very few contaminated drug products that find their way into the marketplace.

Summary

The best way to safeguard patients against adverse reactions caused by bacterial endotoxins is clearly by controlling the quality of the inputs to the process and product and providing processing conditions that restrict the opportunity for microbial proliferation. The long history of improvements in process validation, statistical process control, control of bioburden, and the use of the BET as a tool to monitor the effectiveness of these control procedures have ensured the safety of parenteral products. Therefore, it is not surprising that there have been no reports of product pyrogenicity due to endotoxins with "LER" formulations, which have been in wide use since at least 1987. As evidenced by current data available in the public domain, the "LER" phenomenon is an issue related to the chosen analyte for a particular non-compendial recovery study rather than a concern with <85>, and therefore is neither a release testing nor patient safety issue.

Unfortunately, the historical use of CSEs in the form of highly purified LPS has led to an assumption that these preparations must therefore also be the only or are the most appropriate surrogates for contaminating endotoxin when evaluating pharmaceutical processes and products. Since purified LPS is not and can never be a contaminant in parenteral products, the native CSE mirrors "real life" contamination in a way that chemically extracted and purified RSE and CSE cannot.

The EC recognizes that the study of endotoxin contamination and its implications is a dynamic and complex one, and that the field changes as data are gathered. Information and feedback from the majority of the pharmaceutical/biopharmaceutical scientific community and experts studying endotoxins contamination suggest that broadening analyte options to include a well defined native CSE calibrated

against the RSE as long defined in the USP is not only a good scientific option, but is also an extremely pragmatic one.

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